

# Changes in $\beta$ -1,3-Glucan Synthase Activity in Developing Lima Bean Plants<sup>1</sup>

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## ABSTRACT

A plasma membrane-enriched fraction was isolated from various tissues of developing lima bean seedlings, *Phaseolus lunatus* var Cangreen, to study  $\beta$ -1,3-glucan synthase activity changes. All tissues contained an active  $\beta$ -glucan synthase, including the cotyledons that will be senescent in mature lima bean plants. Young primary leaves exhibited a very active  $\beta$ -glucan synthase; but this activity dropped markedly, about fivefold, as the leaves gained weight and became photosynthetic. Some tissues, such as the hypocotyl and young stem, exhibited an increase in  $\beta$ -glucan synthase activity as the tissues were growing and a decrease as the growth rate slowed. Roots exhibited a high activity early in development that only decreased slightly, about 30%, as root growth increased. Surprisingly the senescent cotyledons contained an activity equivalent to some other tissues that was maintained over our measurement time of 21 days. Perhaps this callose synthesis activity is related to translocation processes as the cotyledons transfer their reserves to the growing seedling. We concluded that  $\beta$ -glucan synthase was not a good indicator of sink strength in these lima bean tissues. The plasma membrane fractions also were tested for other enzymes that might be present because an electron microscope study revealed a low contamination by other types of membranes. The membrane fractions had low but detectable activities of sucrose synthase, UDP-glucose pyrophosphorylase, UDPase, alkaline invertase, and a general phosphatase; but these enzymes exhibited no consistent pattern(s) of activity change with plant development.

Recently, young lima bean seedlings were used as a model to support the thesis that the sucrose synthase pathway, in the cleavage direction, is a major route for supplying both glucose and fructose to intermediary metabolism (27). Sucrose synthase was reported to be a unique enzyme in that it changes its activity more than 10-fold during development. From this and other work, it was proposed that sucrose synthase could be used as a biochemical indicator of sucrose sink strength in specific plant tissues (23, 24). The sucrose synthase pathway required the cycling of both uridylates and PPi (14, 23) to direct UDP-glucose into intermediary metabolism. Many reports (1, 2, 7–9, 25) also have implicated sucrose synthase as

a major enzyme for converting sucrose to UDP-glucose as an entrance step to the synthesis of cell walls, callose, and other plant polysaccharides. Ultimately, we want to test the competition between intermediary metabolism and polysaccharide synthesis at the UDP-glucose branchpoint. As one study, we wished to characterize a nonplastid polysaccharide synthesis activity in developing plant tissues, knowing that the flow of carbon into intermediary metabolism *versus* polysaccharide synthesis likely shifts with the stage of plant development, and we asked if nonplastid polysaccharide synthesis via UDP-glucose might be a component of sink strength in some tissues.

A  $\beta$ -1,3-D-glucan synthase activity in plant plasma membrane preparations that incorporates UDP-[<sup>14</sup>C]glucose into a  $\beta$ -1,3-glucan, callose (12, 13, 17, 19), has been studied in a variety of plant tissues. In contrast, other plant polysaccharide synthesis activities are poorly defined, *e.g.* cellulose synthase. Hence, we chose to study  $\beta$ -glucan synthase even though callose glucan is not normally the major cell wall glucan synthesized by plants; however, it has been suggested that this callose glucan synthase may be an altered form of cellulose synthase (3, 13, 15). The roles of  $\beta$ -glucan synthase in plants are not totally clear, but callose occurs in special cells and at certain cellular sites such as pollen cells, sieve plate pores, and plasmadesmatal channels (3, 4, 16). Seemingly, a function for callose at these sites could be related to cellular sucrose translocation and nutrition. We could not locate published data on  $\beta$ -glucan synthase activity changes during plant development. Therefore, the work presented here was directed at investigating the relationships between the development of young lima bean plant tissues and the activity of  $\beta$ -glucan synthase.

In general, young growth sink tissues *e.g.* young roots, and young leaves, had elevated activities, whereas the  $\beta$ -glucan synthase activities dropped as tissues matured; but all tissues exhibited activity, even including cotyledons. During this investigation, we also became concerned about UDP and UDP-glucose metabolism by other enzymes in these plasma membrane-enriched fractions because we could not find published data on their presence in plasma membrane preparations. Therefore, we also examined these membrane fractions microscopically and assayed the  $\beta$ -glucan synthase-enriched membrane preparations for other enzymes. These results are presented here along with a brief consideration of the roles for  $\beta$ -glucan synthase in plant development.

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## MATERIALS AND METHODS

### Plant Material

Lima bean (*Phaseolus lunatus* var Cangreen) plants were grown in control rooms (Athens, GA) under a regimen of 13 h light, 26°C, and 11 h dark, 18°C, and in charcoal-filtered, evaporative-cooled greenhouses (Riverside, CA) in vermiculite with one-half strength Hoagland's solution supplied at regular intervals. The tissues used in each experiment are identified in the figure.

### Preparation of the Membrane Fraction and Assay of $\beta$ -Glucan Synthase

A plasma membrane fraction with  $\beta$ -glucan synthase activity was isolated from lima bean tissues on a sucrose cushion using the procedures and assay protocol described by Morrow and Lucas (19) with the following exceptions: the resuspending solution contained 20% (w/v) glycerol, which stabilized activity markedly, and 5 mM  $\text{Mg}(\text{C}_2\text{H}_3\text{O}_2)$  was substituted for  $\text{MgCl}_2$  in the assay mixture. The isolated  $\beta$ -glucan synthase fraction maintained original activity for 3 months when stored at  $-80^\circ\text{C}$ , but 30% of the activity was lost within 2 h when stored on an ice bath. Therefore, activity was tested quickly on a fresh preparation or it was stored in liquid  $\text{N}_2$  until assayed. Protein was determined by the Bradford procedure after precipitation with cold 10% (w/v) TCA and decolorization with cold acetone (19).

### Other Enzyme Assays

Sucrose synthase was determined in the cleavage direction (27, 28), with the reaction mixture adjusted to pH 7.5. UDP-glucose was measured spectrophotometrically with UDP-glucose dehydrogenase and  $\text{NAD}^+$  (22). UDP-glucose pyrophosphorylase activity was determined by assay E of Gustafson and Gander (11), except Epps buffer, pH 8.2, was used instead of Tris-HCl. Alkaline invertase, at pH 7.5, was assayed in the sucrose synthase reaction mixture by determining the amount of glucose formed from 5 mM sucrose during the 10 min incubation period by the Sigma glucose [HK] reagent (Sigma

Procedure No. 16-UV, revised November 1986). General phosphatase activity was assayed as inorganic phosphate with the  $\beta$ -glucan synthase reaction mixture following a 10 min incubation period by the method of Leloir and Cardini (18).

### Nucleotide and $\beta$ -Glucan Synthase Product Assays

The  $\beta$ -glucan synthase reaction mixture was also analyzed for UDP-glucose, UTP, UDP, and UMP (6). Uridine compounds were identified by TLC chromatography on PEI-impregnated cellulose plates as described by Verachterst *et al.* (26). UV fluorescence was used to identify these compounds and radioactivity on TLC plates was determined by counting 1 cm sections of the developed plates after supplying  $\text{UDP}[^{14}\text{C}]\text{glucose}$ ,  $[^3\text{H}]\text{UDP}$ , or  $[^3\text{H}]\text{UDP}[^{14}\text{C}]\text{glucose}$  to the  $\beta$ -glucan synthase reaction mixture. Methylation analysis of the reaction product was conducted as previously described (5).

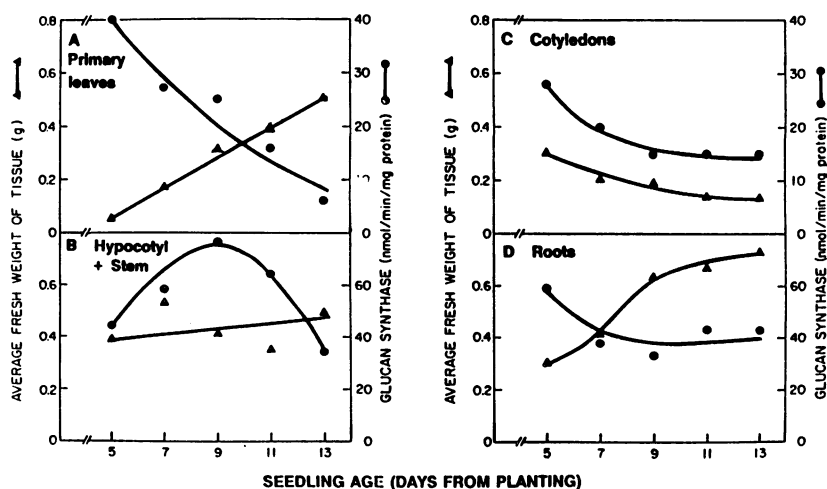
## RESULTS

### Plant Growth and $\beta$ -Glucan Synthase Activity Changes

$\beta$ -Glucan synthase activity of the youngest growing sinks, such as the epicotyls or primary leaves and roots, were highest while these tissues were very small, *e.g.* less than 100 mg average leaf weight, (Fig. 1A, D). The activity then declined, particularly in leaves, to less than one-fifth this maximum activity, by 11 to 13 d (Fig. 1A). In these young seedlings, the hypocotyl and stem tissue exhibited maximum  $\beta$ -glucan synthase activity between 7 and 11 d from germination, with a decline after this period (Fig. 1B). Rather surprising to us, because these dying cotyledons serve as a source tissue, the  $\beta$ -glucan synthase activity in cotyledons was substantial (Fig. 1C). This activity dropped by about one-half with age; but it remained at about the same specific activity in the oldest cotyledons (21 d old) we examined (data not shown). Roots also maintained a high  $\beta$ -glucan synthase activity as the seedlings aged (Fig. 1D). Accurate root growth measurement was not possible beyond 13 d because the roots could not be separated from the vermiculite without excessive loss.

Authenticity of the radioactivity  $\beta$ -glucan product in these

**Figure 1.** Lima bean seedling development, days after planting, and  $\beta$ -glucan synthase activity changes in plasma membrane fractions isolated from various types of seedling tissues. Growth chamber-grown plants (Athens) are used in these studies. The average fresh weight of each tissue and the enzyme activities are from at least three replicates.



tissues was checked by methylation analysis and GC separation; both corresponded with the peak for a (1 $\rightarrow$ 3)glycosidic linkage product.

Older greenhouse-grown plants were used to confirm and extend these relationships between  $\beta$ -glucan synthase activity and tissue age. We note that both the primary leaves and hypocotyl-stem tissues to the primary leaves weighed considerably more in greenhouse plants than in growth chamber plants of similar age. And the dry weights, as a percentage of the total weight for these tissues, were higher (data not given), as expected. During the early growth period, up to 15 d when most comparisons were made, the hypocotyl-stem sections from greenhouse plants did not grow in length as much as the ones from growth chamber plants, but were larger in diameter and contained considerably more dry matter. With greenhouse-grown seedlings, between 17 to 21 d, the average increase in the first trifoliate leaf tissue was more than 300 mg, and the  $\beta$ -glucan synthase decreased from 46 to 19 nmol/min  $\cdot$  mg protein. On day 21, the second trifoliate leaf tissue had an enzyme activity equal to that of the first trifoliate leaf tissue at 17 d old (data not given). Petiole tissues from the first trifoliate leaves (17 to 21 d old) averaged  $88 \pm 2$  nmol/min  $\cdot$  mg protein for the six assays conducted over this period. These cotyledons retained activity up to 2 weeks old. It appears that after leaf tissue becomes a photosynthate source, the level of  $\beta$ -glucan synthase activity declines about four-fifths to a maintenance level, but even the mature leaf maintains activity, whereas stems, petiole, roots, and even cotyledons, which actually were losing weight, (Fig. 1C), continue to maintain a high level of  $\beta$ -glucan synthase as the seedlings mature (Fig. 1). All tissues we studied in lima bean seedlings exhibited  $\beta$ -glucan synthase activity, with petiole and hypocotyl tissues being the most active.

### Characterization of the Membrane Fraction

We assumed that the membrane preparations were primarily plasma membrane preparations, which, in the literature, although they have been used extensively for  $\beta$ -glucan synthase studies (10, 13, 19, 20), have not been routinely examined microscopically nor characterized for other enzymes. Because we wished ultimately to study the metabolism of UDP-glucose and other aspects of the sucrose-synthase pathway, *e.g.* UDP cycling and the roles of PPi, we also examined them microscopically and tested the membrane preparations for other enzymes. Membrane preparations were examined by EM for membrane types using the plasma membrane specific stain, periodic acid, chromic acid, phosphotungstic acid (21) (data not shown). Plasma membranes were the principal membrane fraction present; but the preparations contained a small, readily observable number of golgi stacks and ER vesicles. These observations also caused us to assay for other enzymes that might be present.

### UDP-Glucose Pyrophosphorylase and PPi Inhibition

We had observed that PPi partially inhibited our  $\beta$ -glucan synthase activity. A maximum 30% inhibition was observed over a 10-fold change in UDP-glucose concentration in the reaction mixture with a  $K_i$  of 17  $\mu$ M. One enzyme that would

compete with  $\beta$ -glucan synthase for UDP-glucose, if PPi was present, was UDP-glucose pyrophosphorylase. The rates of UDP-glucose synthesis by this enzyme, in membrane preparations from 11-d-old lima bean hypocotyl and stems above the cotyledons, were  $79 \pm 4$  nmol/min  $\cdot$  mg protein (enzyme assayed in the synthetic direction). This was sufficient to compete with the  $\beta$ -glucan synthase for UDP-glucose in the presence of PPi and likely explains the partial inhibition by PPi.

### UDPase and Uridylate Metabolism

Because of the observed UDP-glucose pyrophosphorylase activity, the  $\beta$ -glucan synthase assay reaction mixture was analyzed for residual UDP-glucose, UTP, UDP, UMP, and Pi after the 10 min reaction time. All of these uridine compounds and Pi were present in the reaction mixture after 10 min of incubation (data not shown). When the substrate for  $\beta$ -glucan synthase was UDP[ $^{14}$ C]glucose, 100% of the  $^{14}$ C label not used in the synthesis of (1 $\rightarrow$ 3)- $\beta$ -D-glucan was recovered as UDP-glucose on the TLC plates (Table I, data not shown).

Additional radioactive compounds, [ $^3$ H]UDP[ $^{14}$ C]glucose and [ $^3$ H]UDP, were used in the  $\beta$ -glucan synthase reaction mixture to test the membrane preparation for UDPase activity. We compared boiled and unboiled membrane preparations using these labeled substrates as given in Table I. With dual label UDP-glucose, and unboiled membrane preparations, the  $^3$ H remaining in UDP-glucose was 23%, with 64% appearing at the  $R_F$  of known UMP and 4% at the  $R_F$  of known UDP. The UMP labeling indicated UDPase activity. Fifty-six percent of the  $^{14}$ C was still present as UDP-glucose, with 20% remaining near the origin. Authentic [ $^{14}$ C]glucose-1-phosphate and [ $^{14}$ C]glucose-6-phosphate, chromatographed in the solvent system used to separate the uridine nucleotides, migrated in a broad band, from the origin to an  $R_F$  of 0.40; hence, the label at lower  $R_F$  values probably were sugar phosphates. The boiled controls show the chromatographic label distribution in the authentic material (Table I).

When [ $^3$ H]UDP was incubated with a boiled membrane preparation, 55% of the  $^3$ H label was recovered at the UDP  $R_F$  (Table I). When authentic [ $^3$ H]UDP was chromatographed, only 61% of the label was at an  $R_F$  of 0.15 (UDP). When the unboiled membrane was incubated with [ $^3$ H]UDP for 10 min, most of the  $^3$ H label was recovered at the  $R_F$  of UMP (Table I). Hence, we concluded from the isotope studies that the plasma membrane preparations contained UDPase.

### Other Enzymes in the Plasma Membrane Fraction

The lima bean plasma membrane fraction also contained a low sucrose synthase activity in extracts from various tissues. The enzyme has a high specific activity in growing sinks, such as young hypocotyl, petiole, stem, and roots tissues. The activity varied from 10 to 34 nmol/min  $\cdot$  mg protein, but with no consistent pattern relative to  $\beta$ -glucan synthase. Low consistent activity was noted in cotyledons,  $<4$  nmol/min  $\cdot$  mg protein. A sucrose synthase reaction mixture, when incubated with membranes prepared from 11-d-old hypocotyl and stem tissue up to the primary leaves, also exhibited alkaline inver-

**Table I.** Recovery of  $^{14}\text{C}$  and  $^3\text{H}$  from Labeled Substrates Used in the  $\beta$ -Glucan Synthase Reaction and the Identification of the Labeled Nucleotides Separated on PEI Cellulose PlatesPlates were first developed in  $\text{H}_2\text{O}$ , then in 0.8 M (LiCl).

Substrate for Reaction <sup>a</sup>	Membrane Fraction Treatment before Reaction	DPM Recovered As	UDP ( $R_f = 0.15$ )	UMP ( $R_f = 0.53$ )	UDPG ( $R_f = 0.73$ )	Front ( $R_f > 0.90$ )
%						
$[^3\text{H}]\text{UDP}[^{14}\text{C}]\text{Glucose}$	Boiled	$^3\text{H}$	1	11	79	6
		$^{14}\text{C}$	1	4	85	10
	Unboiled	$^3\text{H}$	4	64	23	7
		$^{14}\text{C}$	20	8	56	12
$[^3\text{H}]\text{UDP}$	Boiled	$^3\text{H}$	55	4		34
	Unboiled	$^{14}\text{C}$	4	48		36

<sup>a</sup> When  $\text{UDP}[^{14}\text{C}]\text{glucose}$  was the substrate, 100% of the material, not polymerized, was recovered as UDP-glucose.

tase activity but no acid invertase; in the 10 min incubation time,  $0.80 \mu\text{mol}/\text{min} \cdot \text{mg}$  protein (three replicates) of glucose was formed from sucrose. Further work on invertase was not conducted. A general phosphatase activity was measured, in the same membrane fractions and with the  $\beta$ -glucan synthase reaction mixture present, of  $0.12 \text{ nmol}/\text{min} \cdot \text{mg}$  protein (eight replications).

## DISCUSSION

In other research, we have tested several enzymes as potential biochemical indicators of sink strength, knowing that plant tissues grow primarily on sucrose. This work identified sucrose synthase as such an enzyme (27, 28) in developing plant sink tissues. In sucrose metabolism via the sucrose synthase pathway (23, Fig. 1 in Ref. 28), the formation of UDP-glucose is an integral step. Hence, we wished to study the possibility of UDP-glucose also being directed into non-plastid polysaccharide biosynthesis in sink tissues; thus, the present work on  $\beta$ -glucan synthase changes during plant development. Young sink tissues, *i.e.* leaves, roots, hypocotyls (Fig. 1), do have high  $\beta$ -glucan synthase activities. But we found the enzyme in all tissues, even senescing cotyledons (Fig. 1C), and the activity exhibited no consistent pattern with growth weight, *i.e.* with sink strength. For example, in leaves, activity dropped during weight gain; in stems, activity rose and declined during weight gain; in roots, the activity was almost constant during weight gain; and in dying cotyledons, the enzyme just gradually lost activity (Fig. 1). We concluded that  $\beta$ -glucan synthase was not a good enzyme to employ as a biochemical indicator of sink strength in any of these lima bean tissues. But, because the young heterotrophic seedlings were translocating reserves and because callose may have roles in translocation at sieve plates or plasmodesmata (3, 4, 16), further work on the roles of  $\beta$ -glucan synthase in various types of plant tissues seems to be warranted from the results of this developmental study.

Previous reports on plasma membrane preparations from plant tissues working with  $\beta$ -glucan synthase have not referred to assays for other enzyme activities. It became apparent during this study, particularly from microscopy work (not

shown), that the preparations contained a few membranes other than plasma membranes; hence, we assayed for enzyme activities other than  $\beta$ -glucan synthase. We found that  $\text{PPI}$  partially inhibited the incorporation of  $[^{14}\text{C}]\text{glucose}$  from  $\text{UDP}[^{14}\text{C}]\text{glucose}$  into cold alcohol-insoluble glucans. The plasma membrane-enriched preparation contained UDP-glucose pyrophosphorylase, so it was possible that, in the presence of  $\text{PPI}$ , this enzyme was competing for UDP-glucose. In the lima bean preparations, there also was a general phosphatase, a membrane-bound alkaline invertase, and an active  $\text{UDPase}$  (Table I). Morrow and Lucas (20) reported that UDP was an inhibitor of  $\beta$ -glucan synthase prepared from sugar beet petioles. The inhibition, at a high UDP-glucose concentration, was of a competitive type; but at a low substrate concentration, the inhibition was of a different type. Haass *et al.* (12) reported that UDP was a noncompetitive inhibitor of  $\beta$ -glucan synthase prepared from tobacco callus tissue. Neither group of workers tested for the presence of  $\text{UDPase}$ . We concluded that  $\text{UDPase}$  likely was present in the  $\beta$ -glucan synthase fractions and confounded the inhibition studies. However, the function of  $\text{UDPase}$  was not pursued further in this work; although one can easily imagine either a role in the removal of UDP to favor polysaccharide biosynthesis or that  $\text{UDPase}$  simply was in the golgi present in the isolated membranes.

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